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(54) **METHOD OF PREPARING A DOUGH-BASED PRODUCT**

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Related U.S. Application Data

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(60) Provisional application No. 60/614,826, filed on Sep. 30, 2004.

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(51) **Int. Cl.**

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A21D 13/00	(2006.01)

(52) **U.S. Cl.**

CPC ***A21D 8/042*** (2013.01); ***A21D 13/0096*** (2013.01); ***C12N 9/2417*** (2013.01); *C07K 2299/00* (2013.01)

(58) **Field of Classification Search**

CPC C12N 9/2417; A21D 8/042

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

3,026,205	A	3/1962	Stone	
4,500,548	A	2/1985	Silva	
5,409,717	A	4/1995	Apicella	
6,162,628	A	12/2000	Cherry	
6,482,622	B1	11/2002	Cherry	
7,892,806	B2 *	2/2011	Svendsen et al.	435/203
8,361,526	B2 *	1/2013	Beier et al.	426/28

FOREIGN PATENT DOCUMENTS

WO	91/004669	A1	4/1991
WO	99/043794	A1	9/1994
WO	00/029591	A1	5/2000
WO	2006/012899	A1	2/2006

OTHER PUBLICATIONS

Branden et al, 1991, Intro Protein Structure, 247.
Conforti et al, 1998, J Food Qual 21(2), 85-94.
Dauter et al, 1999, Protein Data Bank—3d struc-Novamyl identifiers 1QHO and 1QHP.
Hyun-Ju et al, 1998, Eur J Biochem 253, 251-262.
Rosell et al, 2001, J Agric Food Chem 49(6), 2973-2977.
Sternhagen et al, 1994, Cereal Chem 71(6), 560-563.
Takase et al, 1992, Biochim Biophys Acta 1120, 281-288.
Telloke, 1985, Starch 37(1), 17-22.

* cited by examiner

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(57) **ABSTRACT**

Dough with a high sucrose content (such as cake dough) tends to inhibit the activity of an anti-staling amylase such as Novamyl, making it less effective to prevent the staling of dough-based products with high sucrose content such as cakes. A good anti-staling effect in cakes can be achieved by using a carefully selected anti-staling amylase with certain properties.

Analysis of a 3D structure of Novamyl shows that sucrose may inhibit by binding in the active site. Sucrose docks into the active site of Novamyl differently from the substrate or inhibitor in published models 1QHO and 1QHP. This finding is used to design sucrose-tolerant variants.

11 Claims, 1 Drawing Sheet

ATOM	1	C1	GLC	A	1	39.217	71.096	23.310	1.00	53.03	C
ATOM	2	C2	GLC	A	1	38.281	69.893	23.579	1.00	55.06	C
ATOM	3	O2	GLC	A	1	37.370	70.251	24.614	1.00	58.15	O
ATOM	4	C3	GLC	A	1	39.115	68.665	24.014	1.00	52.01	C
ATOM	5	O3	GLC	A	1	38.239	67.559	24.250	1.00	51.59	O
ATOM	6	C4	GLC	A	1	40.134	68.335	22.918	1.00	53.38	C
ATOM	7	O4	GLC	A	1	41.075	67.354	23.379	1.00	56.10	O
ATOM	8	C5	GLC	A	1	40.910	69.552	22.419	1.00	55.06	C
ATOM	9	O5	GLC	A	1	40.130	70.771	22.258	1.00	55.28	O
ATOM	10	C6	GLC	A	1	41.528	69.246	21.045	1.00	52.65	C
ATOM	11	O6	GLC	A	1	42.190	70.422	20.599	1.00	38.28	O
ATOM	12	H01	GLC	A	1	41.470	68.299	20.510	1.00	52.65	H
ATOM	13	H02	GLC	A	1	38.649	71.976	23.008	1.00	53.03	H
ATOM	14	H03	GLC	A	1	37.734	69.638	22.672	1.00	55.06	H
ATOM	15	H04	GLC	A	1	37.860	70.513	25.396	1.00	58.15	H
ATOM	16	H05	GLC	A	1	39.655	68.880	24.937	1.00	52.01	H
ATOM	17	H06	GLC	A	1	37.543	67.825	24.856	1.00	51.59	H
ATOM	18	H07	GLC	A	1	41.666	67.115	22.661	1.00	56.10	H
ATOM	19	C1	FRU	A	2	39.472	73.740	24.328	1.00	56.70	C
ATOM	20	O1	FRU	A	2	39.013	73.640	22.974	1.00	60.98	O
ATOM	21	C2	FRU	A	2	40.511	72.650	24.697	1.00	60.03	C
ATOM	22	O2	FRU	A	2	39.917	71.335	24.487	1.00	54.05	O
ATOM	23	C3	FRU	A	2	41.038	72.717	26.169	1.00	56.66	C
ATOM	24	O3	FRU	A	2	40.143	72.012	27.049	1.00	57.01	O
ATOM	25	C4	FRU	A	2	42.371	71.945	26.006	1.00	56.00	C
ATOM	26	O4	FRU	A	2	43.252	72.139	27.103	1.00	53.30	O
ATOM	27	C5	FRU	A	2	42.866	72.599	24.701	1.00	54.87	C
ATOM	28	O5	FRU	A	2	41.705	72.741	23.843	1.00	54.62	O
ATOM	29	C6	FRU	A	2	43.903	71.816	23.946	1.00	55.79	C
ATOM	30	O6	FRU	A	2	44.464	72.647	22.938	1.00	54.56	O
ATOM	31	H01	FRU	A	2	43.759	73.046	22.423	1.00	54.56	H
ATOM	32	H02	FRU	A	2	38.615	73.643	24.994	1.00	56.70	H
ATOM	33	H03	FRU	A	2	38.363	74.325	22.804	1.00	60.98	H
ATOM	34	H04	FRU	A	2	41.133	73.718	26.592	1.00	56.66	H
ATOM	35	H05	FRU	A	2	40.267	71.066	26.941	1.00	57.01	H
ATOM	36	H06	FRU	A	2	42.287	70.859	25.973	1.00	56.00	H
ATOM	37	H07	FRU	A	2	42.807	71.896	27.918	1.00	53.30	H
ATOM	38	H08	FRU	A	2	43.345	73.538	24.976	1.00	54.87	H
ATOM	39	H09	FRU	A	2	43.440	70.944	23.487	1.00	55.79	H
ATOM	40	H10	FRU	A	2	44.686	71.486	24.630	1.00	55.79	H
ATOM	41	H11	FRU	A	2	39.962	74.708	24.436	1.00	56.70	H

METHOD OF PREPARING A DOUGH-BASED PRODUCT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 12/964,189 filed Dec. 9, 2010, which is a divisional of U.S. application Ser. No. 11/575,644 filed on Mar. 20, 2007 which is a 35 U.S.C. 371 national application of PCT/DK2005/000602 filed Sep. 23, 2005, which claims priority or the benefit under 35 U.S.C. 119 of Danish application no. PA 2004 01458 filed Sep. 24, 2004 and U.S. provisional application No. 60/614,826 filed Sep. 30, 2004, the contents of which are fully incorporated herein by reference.

SEQUENCE LISTING AND DEPOSITED MICROORGANISMS

Sequence Listing

The present invention comprises a sequence listing.

Deposit of Biological Material

None.

FIELD OF THE INVENTION

The present invention relates to the use of anti-staling amylases in the preparation of dough or dough-based edible products with a high sucrose content.

BACKGROUND OF THE INVENTION

U.S. Pat. No. 3,026,205 describes a process of producing baked confections and the products resuiting therefrom by alpha-amylase.

WO 9104669 describes the use of a maltogenic alpha-amylase to retard the staling of baked products such as bread; the maltogenic alpha-amylase described therein is commercially available under the tradename Novamyl® (product of Novozymes A/S). U.S. Pat. No. 6,162,628 describes Novamyl variants and their use for the same purpose. Three-dimensional structures of Novamyl are published in U.S. Pat. No. 6,162,628 and in the Protein Data Bank (available at http://www.rcsb.org/pdb/) with identifiers 1QHO and 1QHP.

SUMMARY OF THE INVENTION

The inventors have found that a high sucrose content dough (such as cake dough) tends to inhibit the activity of an anti-staling amylases such as Novamyl, making it less effective to prevent the staling of dough-based products with high sucrose content such as cakes. They have found that a good anti-staling effect in cakes can be achieved by using a carefully selected anti-staling amylase with certain properties, and they have identified such amylases.

By analyzing a 3D structure of Novamyl, the inventors further found that sucrose may inhibit by binding in the active site. They have found that sucrose docks into the active site of Novamyl differently from the substrate or inhibitor in published models 1QHO and 1QHP, and they have used this finding to design sucrose-tolerant variants.

Accordingly, the invention provides a method of preparing dough or a dough-based edible product (e.g. a baked product) by adding a sucrose-tolerant anti-staling amylase. It also provides novel sucrose tolerant variants of a maltogenic alpha-amylase.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the cartesian coordinates for the sucrose atoms in this binding configuration, using the coordinate system of the x-ray structure 1QHO.pdb.

DETAILED DESCRIPTION OF THE INVENTION

Maltogenic Alpha-Amylase and Sucrose Docking

A maltogenic alpha-amylase (EC 3.2.1.133) having more than 70% identity (particularly more than 80% or 90%, such as at least 95% or 96% or 97% or 98% or 99%) with the Novamyl sequence shown as SEQ ID NO: 1 may be used as the parent enzyme for designing sucrose tolerant variants. Amino acid identity may be calculated as described in U.S. Pat. No. 6,162,628.

For Novamyl (SEQ ID NO: 1), a 3D structure including a substrate or inhibitor as described in U.S. Pat. No. 6,162,628 or in the Protein Data Bank with the identifier 1QHO or 1QHP may be used. Alternatively, a Novamyl variant may be used, such as a variant described in U.S. Pat. No. 6,162,628 or in this specification, e.g. the variant F188L+D261G+T288P. A 3D structure of a variant may be developed from the Novamyl structure by known methods, e.g. as described in T. L. Blundell et al., Nature, vol. 326, p. 347 ff (26 Mar. 1987); J. Greer, Proteins: Structure, Function and Genetics, 7:317-334 (1990); or Example 1 of WO 9623874.

The inventors found that sucrose may inhibit Novamyl by binding in the active site. Docking of sucrose into the active site of Novamyl (using the software GOLD version 2.1.2, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK and the protein part of the x-ray structure 1QHO.pdb) reveals a specific binding configuration as unique to sucrose. The cartesian coordinates for the sucrose atoms in this binding configuration, using the coordinate system of the x-ray structure 1QHO.pdb are given in FIG. **1**.

Maltogenic Alpha-Amylase Assay

The activity of a maltogenic alpha-amylase may be determined using an activity assay such as the MANU method. One MANU (Maltogenic Amylase Novo Unit) is defined as the amount of enzyme required to release one micro-mole of maltose per minute at a concentration of 10 mg of maltotriose substrate per ml in 0.1 M citrate buffer at pH 5.0, 37° C. for 30 minutes.

Amino Acid Alterations

The amino acid sequence of a maltogenic alpha-amylase may be altered to decrease the sucrose inhibition. The inventors found that the alteration may be made at an amino acid residue having at least one atom within 4 Ångstroms from any of the sucrose atoms when the sucrose molecule is docked in the 3D structure of the maltogenic alpha-amylase. Using the Novamyl structure 1QHO and the sucrose docking in FIG. **1**, the following Novamyl residues are within 4 Å: K44, N86, Y89, H90, Y92, W93, F188, T189, D190, P191, A192, F194, D372, P373, R376.

Further the following positions have been identified as relevant: I15, R81, T87, G88, L196, N371 or N375 of SEQ ID NO: 1.

The alteration may be a substitution or deletion of one or more of the selected residues, or one or more residues (particularly 1-4 residues or 5-6 residues) can be inserted adjacent to a selected residue.

The substitution may be with a smaller or larger residue. A substitution to increase the size of the residue may diminish the space obtained by the docked sucrose molecule thereby preventing the binding of sucrose. Amino acid residues are ranked as follows from smallest to largest: (an equal sign indicates residues with sizes that are practically indistinguishable):

G<A=S=C<V=T<P<L=I=N=D=M<E=Q<K<H<R<F≤Y≤W

The substitution may also be such as to eliminate contacts with the sucrose molecule, in particular by moving or removing potential sites of hydrogen bonding or Van der Waals interactions.

The substitution may particularly be with another residue of the same type where the type is negative, positive, hydrophobic or hydrophilic. The negative residues are D, E, the positive residues are K/R, the hydrophobic residues are A, C, F, G, I, L, M, P, V, W, Y, and the hydrophilic residues are H, N, Q, S, T.

Some particular examples of substitutions are I15T/S/V/L, R18K, K44R/S/T/Q/N, N86Q/S/T, T87N/Q/S, G88A/S/T, Y89W/F/H, H90W/F/Y/R/K/N/Q/M, W93Y/F/M/E/G/V/T/S, F188H/L/I/T/G/V, D190E/Q/G, A192S/T, F194S/L/Y, L196F, N371K/R/F/Y/Q, D372E/Q/S/T/A and N375S/T/D/E/Q.

Examples of deletions are deletion of residue 191 or 192. An example of an insertion is Ala inserted between 192 and 193.

The polypeptide may include other alterations compared to Novamyl (SEQ ID NO: 1), e.g. alterations to increase the thermostability as described in U.S. Pat. No. 6,162,628.

Nomenclature for Amino Acid Alterations

In this specification, an amino acid substitution is described by use of one-letter codes, e.g. K44R. Slashes are used to indicate alternatives, e.g. K44R/S/T/Q/N to indicate substitution of K44 with R or S etc. P191* indicates a deletion of P191. *192aA indicates insertion of one Ala after A192. Commas are used to indicate multiple alterations in the sequence, e.g. F188L, D261G, T288P to indicate a variant with three substitutions.

Properties of Anti-Staling Amylase for Use with Sucrose

The amylase for use in high-sucrose dough may be selected so as to have mainly exo-amylase activity. More specifically, the amylase hydrolyzes amylose so that the average molecular weight of the amylose after 0.4-4% hydrolysis is more than 50% (particularly more than 75%) of the molecular weight before the hydrolysis.

Thus, the amylase may hydrolyze amylose (e.g. wheat amylose or synthetic amylose) so that the average molecular weight of the amylose after 0.4-4% hydrolysis (i.e. between 0.4-4% hydrolysis of the total number of bonds) is more than 50% (particularly more than 75%) of the value before the hydrolysis. The hydrolysis can be conducted in a 1.7% amylose solution by weight at suitable conditions (e.g. 10 minutes at 60° C., pH 5.5), and the molecular weight distribution before and after the hydrolysis can be determined by HPLC. The test may be carried out as described in C. Christophersen et al., Starch 50 (1), 39-45 (1998).

An exo-amylase for use in high-sucrose dough may have a specified sugar tolerance. Compared to its activity in the absence of sucrose, the amylase may have more than 20% activity at 10% sugar, more than 10% activity at 20% sucrose, or more than 4% activity at 40% sucrose. The sugar tolerance may be determined as described in the examples.

The exo-amylase may have optimum activity in the pH range 4.5-8.5. It may have sufficient thermostability to retain at least 20% (particularly at least 40%) activity after 30 minutes incubation at 85° C. at pH 5.7 (50 mM Na-acetate, 1 mM $CaCl_2$) without substrate.

The exo-amylase may be added to the dough in an amount corresponding to 1-100 mg enzyme protein per kg of flour, particularly 5-50 mg per kg.

The exo-amylase may be non-liquefying. This can be determined by letting the exo-amylase act on a 1% wheat starch solution until the reaction is complete, i.e. addition of fresh enzyme causes no further degradation, and analyzing the reaction products, e.g. by HPLC. Typical reaction conditions are e.g. 0.01 mg enzyme per ml starch solution for 48 hours. The exo-amylase is considered non-liquefying if the amount of residual starch after the reaction is at least 20% of the initial amount of starch.

The exo-amylase may have maltogenic alpha-amylase activity (EC 3.2.1.133). The exo-amylase may be the amylase described in DK PA 2004 00021, or it may be a Novamyl variant described in this specification.

Dough and Dough-Based Edible Product

The dough may have a sucrose content above 10% by weight, particularly above 20% or 30%, e.g. 30-40%. The flour content is typically 25-35% by weight of total ingredients. The dough may be made by a conventional cake recipe, typically with cake flour, sugar, fat/oil and eggs as the major ingredients. It may include other conventional ingredients such as emulsifiers, humectants, gums, starch and baking powder. It generally contains such ingredients as soft wheat flour, milk or other liquids, sugar, eggs, chemical leaveners, flavor extracts and spices, as well as others that may or may not include shortening.

The dough is generally heat treated, e.g. by baking or deep frying to prepare an edible product such as cakes including pound cake, yellow and white layer cakes, cakes containing chocolate and cocoa products, sponge cakes, angel food cake, fruit cakes and foam-type cakes and doughnuts.

EXAMPLES

Example 1

Sucrose Tolerance of Novamyl Variants

The amylase activity of a number of polypeptides were tested by incubation with Phadebas tablets (product of Pharmacia®) for 15 minutes at 60° C. in the presence of sucrose at various concentrations (in % by weight). The results are expressed in % of the result without sugar:

Alterations compared to SEQ ID NO: 1	0% sucrose	10% sucrose	20% sucrose	40% sucrose
None	100	13	6	1.5
F188L, D261G, T288P	100	27.5	14.5	6
F194S	100	31.5	18.5	7.5
L196F	100	69	42	23
D190G	100	65	43	21

Example 2

Sucrose Tolerance of Novamyl Variants

A number of polypeptides were tested as in Example 1. The results are expressed as activity with 10% sucrose in % of the activity without sucrose:

Alterations compared to SEQ ID NO: 1	Sugar tolerance
None	15
D261G, T288P	24
F188L, D261G, T288P	35
T288P	56
Y89F, D261G, T288P	42
N86V, F188L, D261G, T288P	37
Y89F, F188L, D261G, T288P	38
Y89H, F188L, D261G, T288P	50
N86T, F188L, D261G, T288P	49
F194S, D261G, T288P	47
L196F	65
D261G, T288P, D372V	62
Q184H, N187D, F194Y	47
D190G	66
N86G, Y89M, F188L, D261G, T288P	47
F188L, D190G, D261G, T288P	68
A192Q, D261G, T288P, S446A	46
F188H	49
P191*	42
A192*	51
A192*, G193*	67
*192aA	44
N86K, F252L, D261G, T288P	49
F194Y, L225S, D261G, T288P	49
F194L, D261G, T288P	54
F194S, D261G, T288P, P642Q	60
D261G, T288P, N375S	58
F188T	37
F188G	36
F188V	41
A192R, F194L, D261G, T288P, G469R	60
A192G, D261G, T288P	41
Y89F, D261G, T288P, I290V, N375S	60

The following variants are also considered of interest in the context of the present invention:

Alterations compared to SEQ ID NO: 1
I15T, N86K, P191S, D261G, T288P
I15T, P191S, D261G, T288P
I15T, P191S, Y258F, D261G, T288P, N375S, Y549C, Q648H
I15T, G153R, P191S, D261G, T288P, N371K, K645R

Example 3

Sucrose Tolerance and Thermostability of Amylases

The following amylases were tested for thermostability and sugar tolerance: bacterial alpha-amylase from *B. amyloliquefaciens* (BAN™, product of Novozymes A/S), fungal alpha-amylase from *A. oryzae* (Fungamyl®, product of Novozymes A/S), maltogenic alpha-amylase having the sequence of SEQ ID NO: 1 (Novamyl®, product of Novozymes A/S), a Novamyl variant having SEQ ID NO: 1 with the substitutions F188L+D261G+T288P, and bacterial alpha-amylase from *B. licheniformis* (Termamyl®, product of Novozymes A/S).

Exo-Amylase Activity

The five amylases were tested for exo-amylase activity as described above. The results show that Novamyl and the Novamyl variant had exo-amylase activity by this test, and the other three did not.

Thermostability

Each amylase was incubated at 85° C. at pH 5.7 (50 mM Na-acetate, 1 mM $CaCl_2$) without substrate, and the amylase activity was measured after 0, 15, 30 and 60 minutes heat treatment. The results are expressed as residual activity in % of the initial activity:

	0	15	30	60
BAN	100	3	1	0
Fungamyl	100	0	0	0
Novamyl	100	51	29	13
Novamyl variant	100	64	48	54
Termamyl	100	100	71	85

The results show that the Novamyl variant and Termamyl were not deactivated by the heat-treatment. BAN and Fungamyl lose all their activity after 15 min while Novamyl loses it gradually with heat-treatment time.

Sucrose Tolerance

The experiment was repeated in 10% sucrose solution. The results are expressed as residual activity in % of the initial activity without sucrose:

	0	15	30	60
BAN	93	2	1	0
Fungamyl	31	0	0	0
Novamyl	7	6	1	3
Novamyl variant	21	19	14	16
Termamyl	116	112	97	82

The results show that BAN and Termamyl were not inhibited by sugar while Fungamyl and the Novamyl variant were somewhat inhibited, and Novamyl was heavily inhibited by sugar. The combination of sugar and heat-treatment shows that the Novamyl variant and Termamyl could be active during baking of cakes. Termamyl and the Novamyl variant fulfill the criterion for thermostability and sugar tolerance used in this invention.

Example 4

Preparation of Sponge Cake with Amylase

Sponge cakes were made with addition of amylase as follows: BAN (0.83. 8.3 or 83 mg/kg flour), Novamyl (1.3 or 13 mg/kg flour) or the Novamyl variant used in Example 1 (1, 10 or 100 mg/kg flour). A control cake was made without amylase.

The cakes were baked according to the High Ratio Sponge Sandwich Cake (HRSSC) method. After baking, the cakes were cooled down for 60-120 minutes, and the cakes were stored at room temperature in sealed plastic bags filled with nitrogen until analysis. The cakes were evaluated on day 1, 3, 7 or 23.

Texture profile analysis (TPA) was performed as described in Bourne M. C. (2002) 2. ed., Food Texture and Viscosity: Concept and Measurement. Academic Press. The results showed that the increase in hardness was slower with increasing dosage of the Novamyl variant. The addition of BAN or Novamyl had only a slight effect, and only at the highest dosage. The cohesiveness of the cakes decreased with storage time. The addition of the Novamyl variant delayed this decrease. The addition of BAN or Novamyl had a slight effect, and only at the highest dosage.

Water mobility was characterized by low field NMR. The addition of the Novamyl variant and BAN increased the mobility, indicating that the two amylases were able to keep the cakes more moist. Novamyl had virtually no effect.

A small sensory evaluation of softness and moistness was performed on day 13 for the 3 cakes with the Novamyl variant and the control cake. The cakes were evaluated regarding three parameters; Firmness, Moistness and preferability. The control was the firmest, driest and least preferred. The higher dosage of the Novamyl variant, the less firm (softer), moister and better liked.

A large panel sensory evaluation was performed on day 13. It was a paired comparison test where a control cake was compare to the cake with the Novamyl variant at the highest dosage. A 30-member panel was asked two questions (1) Which cake is moister and (2) which cake is fresher. All panel members agreed on that the cake with the Novamyl variant was moister and fresher. The preference was significant at a significance level above 99.999%.

To summarize, the data show that the Novamyl variant had anti-staling properties and was able to improve moistness perception and moistness measured by NMR. The two other amylases had only a slight effect.

Example 5

High-Ratio Unit Cakes

Cakes were made with addition of amylase as follows: BAN (0.83. 8.3 or 83 mg/kg flour) or the Novamyl variant used in Example 1 (1, 10 or 100 mg/kg flour). A control cake was made without amylase.

Cakes were baked according to the High ratio unit cake (HRUC) method. After baking, the cakes were cooled down for 60-120 minutes, and the cakes were stored at room temperature in sealed plastic bags filled with Nitrogen until analysis. The cakes were evaluated on day 7, 20 and 34 by the same methods as in the previous example.

The increase in hardness was slower with the Novamyl variant at the highest dosage. The addition of BAN to the cake resulted in a low volume and a doughy cake which gave poor results in hardness measurements.

The addition of the Novamyl variant delayed the decrease in cohesiveness while BAN did not influence it at all.

The Novamyl variant and BAN were able to keep the cake more moist than the control. This increase in mobility of the free water could partly be explained by the cakes with BAN and the Novamyl variant being able to retain the moisture content.

A small sensory evaluation on day 34 showed that the cake with the Novamyl variant at the highest dosage was clearly better than the control cake; it was more moist and it was less crumbly.

Over-all, there was an anti-staling effect of the Novamyl variant at the high dosage, similar to the effect on sponge cakes in the previous example. The staling of HRUC cakes was slower than Sponge cakes but it was still evident that the Novamyl variant had an anti-staling effect. The anti-staling effect was seen with texture analysis, NMR and sensory evaluation. BAN showed anti-staling effects in HRUC but it was sensitive to over-dosage which resulted in cake collapse and a doughy cake.

Example 6

Sponge Cake

Sponge cakes were made with addition of the amylase of DK PA 2004 00021 at dosages 0.5, 1, 2, 5 and 20 mg/kg flour and a control cake without amylase.

Texture and NMR was measured on day 1, 7 and 13. The addition of the amylase reduced the increase in firmness, especially at the highest dosage. The amylase also had a beneficial effect on the mobility of water which was correlated with the moistness of the cake.

A blind sensory ranking evaluation performed on day 14 showed a ranking according to the dosage, the higher dosage the more soft and moist cake. The most preferred cake was the one with the highest dosage.

Example 7

Baking Procedure Tegral Allegro Cake

Recipe

The following recipe was used:

	%
Tegral Allegro mix*	100
Pasteurized whole egg	50
Butter	50
Enzymes	According to trial. 0 or 25 mg/kg flour.

*commercially available from Puratos NV/SA, Groot-Bijgaarden, Belgium

Procedure

The ingredients were scaled into a mixing bowl and mixed using an industrial mixer (e.g. Bjørn AR 5 A Varimixer) with a suitable paddle speed. 300 g of the dough was poured into forms. The cakes are baked in a suitable oven (e.g. Sveba Dahlin deck oven) for 45 min. at 180° C. The cakes were allowed to cool down at room temperature for 1 hour.

The volume of the cakes was determined when the cakes had cooled down using the rape seed displacement method. The cakes were packed under nitrogen in sealed plastic bags and stored at room temperature until analysis.

The cakes were evaluated on day 1, 7 and 14, two cakes were used at each occasions.

The cohesiveness and hardness of the cakes was evaluated with Texture analyser and the water mobility was characterized by low field NMR.

The Texture profile analysis (TPA) was performed as described in Bourne M. C. (2002) 2. ed., Food Texture and Viscosity: Concept and Measurement. Academic Press.

The mobility of free water was determined as described by P. L. Chen, Z. Long, R. Ruan and T. P. Labuza, Nuclear Magnetic Resonance Studies of water Mobility in Bread during Storage. Lebensmittel Wissenschaft and Technologie 30, 178-183 (1997). The mobility of free water has been described in literature to correlate to moistness of bread crumb.

Result

Compared to cakes with no addition of enzymes the volume of the cakes is not affected by the addition of the reference enzyme (SEQ ID NO.: 1) nor by the addition of variants hereof, i.e. the cakes did not collapse upon addition of enzyme.

The cohesiveness of the cakes decreased with storage time. The addition of variants of SEQ ID NO: 1 delayed this decrease as can be seen in Table 1.

TABLE 1

Change in Cohesiveness [gs/gs] with storage time of cakes with 25 mg protein enzyme per kg flour

Enzyme	Day 1	Day 7	Day 14
No enzyme	0.44	0.35	0.32
Seq ID No: 1	0.43	0.38	0.36
F188L, D261G, T288P	0.46	0.42	0.41

TABLE 1-continued

Change in Cohesiveness [gs/gs] with storage time of cakes with 25 mg protein enzyme per kg flour			
Enzyme	Day 1	Day 7	Day 14
Y89F, D261G, T288P	0.45	0.43	0.39
N86G, Y89M, F188L, D261G, T288P	0.44	0.42	0.38
T288P	0.44	0.40	0.41
F194S, D261G, T288P	0.47	0.43	0.42
D261G, T288P, D372V	0.46	0.43	0.37
A192Q, D261G, T288P, S446A	0.44	0.42	0.39
A192R, F194L, D261G, T288P, G469R	0.47	0.44	0.42
A192G, D261G, T288P	0.46	0.42	0.39
N86K, F252L, D261G, T288P	0.45	0.41	0.39
F194L, D261G, T288P	0.45	0.42	0.42
F194S, D261G, T288P, P642Q	0.44	0.40	0.39
Y89F, D261G, T288P, I290V, N375S	0.43	0.42	0.40

The free water mobility is correlated with the moist perception of the cake crumb, it decreases with time. The addition of the Novamyl variants increased the mobility compared to the control, indicating that the amylases were able to keep the cakes more moist. Results are listed in Table 2.

TABLE 2

Change in free water mobility [micros] with storage time of cakes with 25 mg protein enzyme per kg flour			
Enzyme	Day 1	Day 7	Day 14
No enzyme	7077	5111	4175
Seq ID No: 1	6990	5460	4583
F188L, D261G, T288P	7216	5624	4656
Y89F, D261G, T288P	7085	6044	5151
N86G, Y89M, F188L, D261G, T288P	7493	5349	5120
T288P	7458	5785	4858
F194S, D261G, T288P	7746	6373	5325
D261G, T288P, D372V	7417	5517	4525

TABLE 2-continued

Change in free water mobility [micros] with storage time of cakes with 25 mg protein enzyme per kg flour			
Enzyme	Day 1	Day 7	Day 14
A192Q, D261G, T288P, S446A	7357	5714	5041
A192R, F194L, D261G, T288P, G469R	7549	5536	no data
A192G, D261G, T288P	7546	5815	no data
N86K, F252L, D261G, T288P	7349	5295	4775
F194L, D261G, T288P	7773	6803	5750
F194S, D261G, T288P, P642Q	8152	5969	4971
Y89F, D261G, T288P, I290V, N375S	7753	6175	4811

The hardness of the cakes increased with storage time. The addition of variants of SEQ ID NO: 1 delayed this increase in hardness as can be seen in Table 3.

TABLE 3

Change in hardness [g] with storage time of cakes with 25 mg protein enzyme per kg flour			
Enzyme	Day 1	Day 7	Day 14
No enzyme	647	1060	1408
Seq ID No: 1	677	997	1171
F188L, D261G, T288P	683	951	1167
Y89F, D261G, T288P	649	998	1160
N86G, Y89M, F188L, D261G, T288P	630	844	1194
T288P	719	1101	1098
F194S, D261G, T288P	672	943	1061
D261G, T288P, D372V	593	962	1344
A192Q, D261G, T288P, S446A	680	931	1159
A192R, F194L, D261G, T288P, G469R	720	987	1209
A192G, D261G, T288P	707	1024	1102
N86K, F252L, D261G, T288P	678	955	1248
F194L, D261G, T288P	648	895	1050
F194S, D261G, T288P, P642Q	674	1028	1316
Y89F, D261G, T288P, I290V, N375S	602	731	827

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                         SEQUENCE LISTING


<160> NUMBER OF SEQ ID NOS: 1

<210> SEQ ID NO 1
<211> LENGTH: 686
<212> TYPE: PRT
<213> ORGANISM: Bacillus stearothermophilus

<400> SEQUENCE: 1

Ser Ser Ser Ala Ser Val Lys Gly Asp Val Ile Tyr Gln Ile Ile Ile
1               5                   10                  15

Asp Arg Phe Tyr Asp Gly Asp Thr Thr Asn Asn Asn Pro Ala Lys Ser
            20                  25                  30

Tyr Gly Leu Tyr Asp Pro Thr Lys Ser Lys Trp Lys Met Tyr Trp Gly
        35                  40                  45

Gly Asp Leu Glu Gly Val Arg Gln Lys Leu Pro Tyr Leu Lys Gln Leu
    50                  55                  60

Gly Val Thr Thr Ile Trp Leu Ser Pro Val Leu Asp Asn Leu Asp Thr
65                  70                  75                  80

Leu Ala Gly Thr Asp Asn Thr Gly Tyr His Gly Tyr Trp Thr Arg Asp
                85                  90                  95

Phe Lys Gln Ile Glu Glu His Phe Gly Asn Trp Thr Thr Phe Asp Thr
            100                 105                 110

Leu Val Asn Asp Ala His Gln Asn Gly Ile Lys Val Ile Val Asp Phe
        115                 120                 125
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Val Pro Asn His Ser Thr Pro Phe Lys Ala Asn Asp Ser Thr Phe Ala
    130                 135                 140

Glu Gly Gly Ala Leu Tyr Asn Asn Gly Thr Tyr Met Gly Asn Tyr Phe
145                 150                 155                 160

Asp Asp Ala Thr Lys Gly Tyr Phe His His Asn Gly Asp Ile Ser Asn
                165                 170                 175

Trp Asp Asp Arg Tyr Glu Ala Gln Trp Lys Asn Phe Thr Asp Pro Ala
            180                 185                 190

Gly Phe Ser Leu Ala Asp Leu Ser Gln Glu Asn Gly Thr Ile Ala Gln
        195                 200                 205

Tyr Leu Thr Asp Ala Ala Val Gln Leu Val Ala His Gly Ala Asp Gly
    210                 215                 220

Leu Arg Ile Asp Ala Val Lys His Phe Asn Ser Gly Phe Ser Lys Ser
225                 230                 235                 240

Leu Ala Asp Lys Leu Tyr Gln Lys Lys Asp Ile Phe Leu Val Gly Glu
                245                 250                 255

Trp Tyr Gly Asp Asp Pro Gly Thr Ala Asn His Leu Glu Lys Val Arg
            260                 265                 270

Tyr Ala Asn Asn Ser Gly Val Asn Val Leu Asp Phe Asp Leu Asn Thr
        275                 280                 285

Val Ile Arg Asn Val Phe Gly Thr Phe Thr Gln Thr Met Tyr Asp Leu
    290                 295                 300

Asn Asn Met Val Asn Gln Thr Gly Asn Glu Tyr Lys Tyr Lys Glu Asn
305                 310                 315                 320

Leu Ile Thr Phe Ile Asp Asn His Asp Met Ser Arg Phe Leu Ser Val
                325                 330                 335

Asn Ser Asn Lys Ala Asn Leu His Gln Ala Leu Ala Phe Ile Leu Thr
            340                 345                 350

Ser Arg Gly Thr Pro Ser Ile Tyr Tyr Gly Thr Glu Gln Tyr Met Ala
        355                 360                 365

Gly Gly Asn Asp Pro Tyr Asn Arg Gly Met Met Pro Ala Phe Asp Thr
    370                 375                 380

Thr Thr Thr Ala Phe Lys Glu Val Ser Thr Leu Ala Gly Leu Arg Arg
385                 390                 395                 400

Asn Asn Ala Ala Ile Gln Tyr Gly Thr Thr Thr Gln Arg Trp Ile Asn
                405                 410                 415

Asn Asp Val Tyr Ile Tyr Glu Arg Lys Phe Phe Asn Asp Val Val Leu
            420                 425                 430

Val Ala Ile Asn Arg Asn Thr Gln Ser Ser Tyr Ser Ile Ser Gly Leu
        435                 440                 445

Gln Thr Ala Leu Pro Asn Gly Ser Tyr Ala Asp Tyr Leu Ser Gly Leu
    450                 455                 460

Leu Gly Gly Asn Gly Ile Ser Val Ser Asn Gly Ser Val Ala Ser Phe
465                 470                 475                 480

Thr Leu Ala Pro Gly Ala Val Ser Val Trp Gln Tyr Ser Thr Ser Ala
                485                 490                 495

Ser Ala Pro Gln Ile Gly Ser Val Ala Pro Asn Met Gly Ile Pro Gly
            500                 505                 510

Asn Val Val Thr Ile Asp Gly Lys Gly Phe Gly Thr Thr Gln Gly Thr
        515                 520                 525

Val Thr Phe Gly Gly Val Thr Ala Thr Val Lys Ser Trp Thr Ser Asn
    530                 535                 540
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Arg Ile Glu Val Tyr Val Pro Asn Met Ala Ala Gly Leu Thr Asp Val
545                 550                 555                 560

Lys Val Thr Ala Gly Gly Val Ser Ser Asn Leu Tyr Ser Tyr Asn Ile
                565                 570                 575

Leu Ser Gly Thr Gln Thr Ser Val Val Phe Thr Val Lys Ser Ala Pro
            580                 585                 590

Pro Thr Asn Leu Gly Asp Lys Ile Tyr Leu Thr Gly Asn Ile Pro Glu
        595                 600                 605

Leu Gly Asn Trp Ser Thr Asp Thr Ser Gly Ala Val Asn Asn Ala Gln
    610                 615                 620

Gly Pro Leu Leu Ala Pro Asn Tyr Pro Asp Trp Phe Tyr Val Phe Ser
625                 630                 635                 640

Val Pro Ala Gly Lys Thr Ile Gln Phe Lys Phe Phe Ile Lys Arg Ala
                645                 650                 655

Asp Gly Thr Ile Gln Trp Glu Asn Gly Ser Asn His Val Ala Thr Thr
            660                 665                 670

Pro Thr Gly Ala Thr Gly Asn Ile Thr Val Thr Trp Gln Asn
        675                 680                 685
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The invention claimed is:

1. A method of preparing a high-sucrose dough or a high-sucrose dough-based edible product, comprising adding an enzyme having maltogenic alpha-amylase activity to the dough in an amount corresponding to 1-100 mg enzyme protein per kg of flour, wherein the dough comprises at least 10% sucrose by weight, and the enzyme:

a) has an amino acid sequence which is at least 95% identical to SEQ ID NO: 1,

b) compared to SEQ ID NO: 1 comprises an alteration at a position comprising F188L and D261G; and

c) has more than 20% maltogenic alpha-amylase activity at 10% sucrose by weight compared to its maltogenic alpha-amylase activity in the absence of sucrose.

2. The method of claim **1**, wherein the polypeptide is at least 97% identical to SEQ ID NO: 1.

3. The method of claim **1**, wherein the polypeptide is at least 98% identical to SEQ ID NO: 1.

4. The method of claim **1**, wherein the polypeptide is at least 99% identical to SEQ ID NO: 1.

5. The method of claim **1**, wherein the polypeptide has the amino acid sequence of SEQ ID NO: 1 with one of the following sets of alterations:

F188L,D261G,T288P
N86V,F188L,D261G,T288P
Y89F,F188L,D261G,T288P
Y89H,F188L,D261G,T288P
N86T,F188L,D261G,T288P
N86G,Y89M, F188L,D261G,T288P
F188L,D190G,D261G,T288P

6. The method of claim **1**, wherein the polypeptide further comprises an amino acid alteration which is substitution or deletion of or insertion adjacent to 115, R18, K44, N86, T87, G88, Y89, H90, Y92, W93, T189, D190, P191, A192, F194, L196, D329, N371, D372, P373, N375or R376.

7. The method of claim **6**, wherein the alteration is substitution with a larger or smaller amino acid residue.

8. The method of claim **6**, wherein the alteration is insertion of 1-4 amino acid residues at the N- or C-side of the specified residue.

9. The method of claim **6**, wherein the polypeptide further comprises a substitution 115T/S/V/L, R18K, K44R/S/T/Q/N, N86Q/S/T, T87N/Q/S, G88A/S/T, Y89W/F/H, H90W/FN/R/K/N/Q/M, W93Y/F/M/E/G/V/T/S, D190E/Q/G, A192G/S/T/Q/R, F194S/LN, L196F, N371K/R/FN/Q or D372E/Q/S/T/A, a deletion of 191 or 192 or an insertion of Ala after 192.

10. The method of claim **1**, wherein the enzyme has more than 10% maltogenic alpha-amylase activity at 20% sucrose by weight.

11. The method of claim **1**, wherein the enzyme has more than 4% maltogenic alpha-amylase activity at 40% sucrose by weight.

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